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METHODS FOR DELIVERING DNA TO MUSCLE CELLS
USING RECOMBINANT ADENO-ASSOCIATED VIRUS VECTORS

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Description

Technical Field

The present invention relates generally to DNA
15 delivery methods. More particularly, the invention
relates to the use of recombinant adeno-associated virus
(AAV) virions for delivery of a selected gene to muscle
cells and tissue. The method provides for sustained,
high-level expression of the delivered gene.

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Background of the Invention

Gene delivery is a promising method for the
treatment of acquired and inherited diseases.
Muscle tissue is an appealing gene delivery target
25 because it is readily accessible, well-differentiated and
nondividing. Barr and Leiden (1991) *Science* 254:1507-
1509. These properties are important in the selection of
appropriate delivery strategies to achieve maximal gene
transfer.

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Several experimenters have demonstrated the
ability to deliver genes to muscle cells with the
subsequent systemic appearance of proteins encoded by the
delivered genes. See, e.g., Wolff et al. (1990) *Science*
247:1465-1468; Acsadi et al. (1991) *Nature* 352:815-818;
35 Barr and Leiden (1991) *Science* 254:1507-1509; Dhawan et

al. (1991) *Science* 254:1509-1512; Wolff et al. (1992) *Human Mol. Genet.* 1:363-369; Eyal et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:4523-4527; Davis et al. (1993) *Hum. Gene Therapy* 4:151-159.

5 Genes have been delivered to muscle by direct injection of plasmid DNA, such as described by Wolff et al. (1990) *Science* 247:1465-1468; Acsadi et al. (1991) *Nature* 352:815-818; Barr and Leiden (1991) *Science* 254:1507-1509. However, this mode of administration
10 generally results in sustained but low levels of expression. Low but sustained expression levels may be effective in certain situations, such as for providing immunity.

Viral based systems have also been used for
15 gene delivery to muscle. For example, human adenoviruses are double-stranded DNA viruses which enter cells by receptor-mediated endocytosis. These viruses have been considered well suited for gene transfer because they are easy to grow and manipulate and they exhibit a broad host
20 range *in vivo* and *in vitro*. Adenoviruses are able to infect quiescent as well as replicating target cells and persist extrachromosomally, rather than integrating into the host genome.

Despite these advantages, adenovirus vectors
25 suffer from several drawbacks which make them ineffective for long term gene therapy. In particular, adenovirus vectors express viral proteins that may elicit an immune response which may decrease the life of the transduced cell. This immune reaction may preclude subsequent
30 treatments because of humoral and/or T cell responses. Furthermore, the adult muscle cell may lack the receptor which recognizes adenovirus vectors, precluding efficient transduction of this cell type using such vectors. Thus, attempts to use adenoviral vectors for the delivery
35 of genes to muscle cells has resulted in poor and/or

3

transitory expression. See, e.g., Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Acsadi et al. (1994) *Hum. Mol. Genetics* 3:579-584; Acsadi et al. (1994) *Gene Therapy* 1:338-340; Dai et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:1401-1405; Descamps et al. (1995) *Gene Therapy* 2:411-417; Gilgenkrantz et al. (1995) *Hum. Gene Therapy* 6:1265-1274.

Gene therapy methods based upon surgical transplantation of myoblasts has also been attempted. See, e.g., International Publication no. WO 95/13376; Dhawan et al. (1991) *Science* 254:1509-1512; Wolff et al. (1992) *Human Mol. Genet.* 1:363-369; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hamamori et al. (1994) *Hum. Gene Therapy* 5:1349-1356; Hamamori et al. (1995) *J. Clin. Invest.* 95:1808-1813; Blau and Springer (1995) *New Eng. J. Med.* 333:1204-1207; Leiden, J.M. (1995) *New Eng. J. Med.* 333:871-872; Mendell et al. (1995) *New Eng. J. Med.* 333:832-838; and Blau and Springer (1995) *New Eng. J. Med.* 333:1554-1556. However, such methods require substantial tissue culture manipulation and surgical expertise, and, at best, show inconclusive efficacy in clinical trials. Thus, a simple and effective method of gene delivery to muscle, resulting in long-term expression of the delivered gene, would be desirable.

Recombinant vectors based on adeno-associated viruses (AAV) have been used for DNA delivery. AAV is a helper-dependent DNA parvovirus which belongs to the genus *Dependovirus*. AAV requires infection with an unrelated helper virus, such as adenovirus, a herpesvirus or vaccinia, in order for a productive infection to occur. The helper virus supplies accessory functions that are necessary for most steps in AAV replication. In the absence of such infection, AAV establishes a latent state by insertion of its genome into a host cell

chromosome. Subsequent infection by a helper virus rescues the integrated copy which can then replicate to produce infectious viral progeny. AAV has a wide host range and is able to replicate in cells from any species so long as there is also a successful infection of such cells with a suitable helper virus. Thus, for example, human AAV will replicate in canine cells coinfecting with a canine adenovirus. AAV has not been associated with any human or animal disease and does not appear to alter the biological properties of the host cell upon integration. For a review of AAV, see, e.g., Berns and Bohenzky (1987) *Advances in Virus Research* (Academic Press, Inc.) 32:243-307.

The AAV genome is composed of a linear, single-stranded DNA molecule which contains approximately 4681 bases (Berns and Bohenzky, *supra*). The genome includes inverted terminal repeats (ITRs) at each end which function in *cis* as origins of DNA replication and as packaging signals for the virus. The internal nonrepeated portion of the genome includes two large open reading frames, known as the AAV rep and cap regions, respectively. These regions code for the viral proteins involved in replication and packaging of the virion. For a detailed description of the AAV genome, see, e.g., Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129.

The construction of recombinant AAV (rAAV) virions has been described. See, e.g., U.S. Patent Nos. 5,173,414 and 5,139,941; International Publication Numbers WO 92/01070 (published 23 January 1992) and WO 93/03769 (published 4 March 1993); Lebkowski et al. (1988) *Molec. Cell. Biol.* 8:3988-3996; Vincent et al. (1990) *Vaccines 90* (Cold Spring Harbor Laboratory Press); Carter, B.J. (1992) *Current Opinion in Biotechnology* 3:533-539; Muzyczka, N. (1992) *Current Topics in*

Microbiol. and Immunol. 158:97-129; and Kotin, R.M.
(1994) *Human Gene Therapy* 5:793-801.

Recombinant AAV virion production generally involves cotransfection of a host cell with an AAV vector plasmid and a helper construct which provides AAV helper functions to complement functions missing from the AAV vector plasmid. In this manner, the host cell is capable of expressing the AAV proteins necessary for AAV replication and packaging. The AAV vector plasmid will include the DNA of interest flanked by AAV ITRs which provide for AAV replication and packaging functions. AAV helper functions can be provided via an AAV helper plasmid that includes the AAV rep and/or cap coding regions but which lacks the AAV ITRs. Accordingly, the helper plasmid can neither replicate nor package itself. The host cell is then infected with a helper virus to provide accessory functions, or with a vector which includes the necessary accessory functions. The helper virus transactivates the AAV promoters present on the helper plasmid that direct the transcription and translation of AAV rep and cap regions. Upon subsequent culture of the host cells, recombinant AAV virions harboring the DNA of interest, are produced.

Recombinant AAV virions have been shown to exhibit tropism for respiratory epithelial cells (Flotte et al. (1992) *Am. J. Respir. Cell Mol. Biol.* 7:349-356; Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790; Flotte et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:10613-10617) and neurons of the central nervous system (Kaplitt et al. (1994) *Nature Genetics* 8:148-154). These cell types are well-differentiated, slowly-dividing or postmitotic. Flotte et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:10613-10617; Kaplitt et al. (1994) *Nature Genetics* 8:148-154. The ability of AAV vectors to transduce nonproliferating cells (Podsakoff et al. (1994) *J. Virol.* 68:5656-5666;

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Russell et al. (1994) *Proc. Natl. Acad. Sci. USA*
91:8915-8919; Flotte et al. (1994) *Am. J. Respir. Cell*
Mol. Biol. 11:517-521) along with the attributes of being
inherently defective and nonpathogenic, place AAV in a
5 unique position among gene therapy viral vectors.

Despite these advantages, the use of
recombinant AAV virions to deliver genes to muscle cells
in vivo has not heretofore been disclosed.

10 Summary of the Invention

Accordingly, the present invention is based on
the surprising and unexpected discovery that recombinant
AAV (rAAV) virions provide for efficient delivery of
genes and sustained production of therapeutic proteins in
15 various muscle cell types. The invention allows for in
vivo secretion of the therapeutic protein from transduced
muscle cells such that systemic delivery is achieved.
These results are seen with both in vivo and in vitro
modes of DNA delivery. Hence, rAAV virions allow
20 delivery of DNA directly to muscle tissue. The ability
to deliver and express genes in muscle cells, as well as
to provide for secretion of the produced protein from
transduced cells, allows the use of gene therapy
approaches to treat and/or prevent a wide variety of
25 disorders.

Furthermore, the ability to deliver DNA to
muscle cells by intramuscular administration in vivo
provides a more efficient and convenient method of gene
transfer.

30 Thus, in one embodiment, the invention relates
to a method of delivering a selected gene to a muscle
cell or tissue. The method comprises:

(a) providing a recombinant AAV virion which
comprises an AAV vector, the AAV vector comprising the
35 selected gene operably linked to control elements capable

7

of directing the *in vivo* transcription and translation of the selected gene; and

(b) introducing the recombinant AAV virion into the muscle cell or tissue.

5 In particularly preferred embodiments, the selected gene encodes a therapeutic protein, such as erythropoietin.

In another embodiment, the invention is directed to a muscle cell or tissue transduced with a
10 recombinant AAV virion which comprises an AAV vector, the AAV vector comprising a selected gene operably linked to control elements capable of directing the *in vivo* transcription and translation of the selected gene.

In still further embodiments, the invention is
15 directed to a method of treating an acquired or inherited disease in a mammalian subject comprising introducing into a muscle cell or tissue of the subject, *in vivo*, a therapeutically effective amount of a pharmaceutical composition which comprises (a) a pharmaceutically
20 acceptable excipient; and (b) recombinant AAV virions. The recombinant AAV virions comprise an AAV vector, the AAV vector comprising a selected gene operably linked to control elements capable of directing the transcription and translation of the selected gene when present in the
25 subject.

In yet another embodiment, the invention is directed to a method of treating an acquired or inherited disease in a mammalian subject comprising:

(a) introducing a recombinant AAV virion into a
30 muscle cell or tissue *in vitro* to produce a transduced muscle cell. The recombinant AAV virion comprises an AAV vector, the AAV vector comprising a selected gene operably linked to control elements capable of directing the transcription and translation of the selected gene
35 when present in the subject; and

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(b) administering to the subject a therapeutically effective amount of a composition comprising a pharmaceutically acceptable excipient and the transduced muscle cells from step (a).

5 In a further embodiment, the invention relates to a method for delivering a therapeutically effective amount of a protein systemically to a mammalian subject comprising introducing into a muscle cell or tissue of the subject a pharmaceutical composition which comprises
10 (a) a pharmaceutically acceptable excipient; and (b) recombinant AAV virions, wherein the recombinant AAV virions comprise an AAV vector, the AAV vector comprising a selected gene operably linked to control elements capable of directing the transcription and translation of
15 the selected gene when present in the subject, wherein the introducing is done *in vivo*.

In another embodiment, the invention is directed to a method for delivering a therapeutically effective amount of a protein systemically to a mammalian
20 subject comprising:

(a) introducing a recombinant AAV virion into a muscle cell or tissue *in vitro* to produce a transduced muscle cell, wherein the recombinant AAV virion comprises an AAV vector, the AAV vector comprising a selected gene
25 operably linked to control elements capable of directing the transcription and translation of the selected gene when present in the subject; and

(b) administering to the subject a therapeutically effective amount of a composition
30 comprising a pharmaceutically acceptable excipient and the transduced muscle cells from step (a).

In other embodiments, the invention is directed to an AAV vector comprising a gene encoding human erythropoietin (hEPO) operably linked to control elements
35 capable of directing the *in vivo* transcription and

translation of the gene, as well as a recombinant AAV (rAAV) virion comprising the vector.

These and other embodiments of the subject invention will readily occur to those of ordinary skill
5 in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 shows the dose-response of rAAV-LacZ expression in Balb/c mice tibialis anterior muscle in
10 vivo, as described in Example 1. Adult Balb/c mice were injected intramuscularly (IM) with various doses of rAAV-LacZ vector. At 2 and 8 weeks post injection, tissue was harvested for analysis of beta-galactosidase (β -gal). B-gal expression was analyzed by measurement of
15 relative light units (RLU) emitted from muscle homogenates detected by a luminometer using the Galacto-Light™ reagent detection kit.

Figure 2 shows the time course of expression of rAAV-LacZ vector in Balb/c mice tibialis anterior muscle
20 in vivo as described in Example 2. Adult Balb/c mice were injected with 8×10^9 vector genomes of rAAV-LacZ IM and muscle was harvested and assessed for total β -gal expression representing various time points post-injection. B-gal expression was analyzed as
25 described above.

Figure 3 shows the secretion of human erythropoietin (hEPO) from transduced myotubes and myoblasts, as described in Example 3. Myotubes (differentiated cells) or myoblasts (actively dividing
30 cells) were transduced with rAAV-hEPO at a ratio of approximately 10^5 vector particles per target cell. Subsequently 24-hour levels of hEPO were analyzed in supernatants at various time points. Baseline levels of hEPO, prior to transduction, were below the level of

detection in both cell populations; the values at each point represent replicate values +/- standard deviation.

Figure 4 shows sustained expression of hEPO resulting in elevated hematocrit in Balb/c mice, *in vivo*, as described in Example 4. Animals were injected IM in their hindlimbs percutaneously with 6.5×10^{11} vector genomes, and serum levels of hEPO and measurements of hematocrit (HCT) were analyzed. Each point is a replicate value, with error bars indicating standard deviation.

Figure 5 shows dose-response curves for rAAV-hEPO in Balb/c mice IM at 20 and 41 days post-injection, as described in Example 4. These data indicate that there is a linear dose response of serum hEPO levels at both time points. Each point is a replicate value ($n=4$), with error bars indicating standard deviation.

Figure 6 depicts an *in vivo* comparison of circulating hEPO levels obtained with IM rAAV-hEPO vector versus IM pAAV-hEPO plasmid at days 20 and 41 post-injection, as described in Example 4. Either 3×10^{11} single-stranded vector genomes or 1.4×10^{13} double-stranded plasmid molecules present in $100\mu\text{g}$ of plasmid DNA were injected IM in Balb/c mice. On days 20 and 41, serum hEPO levels were measured by the R and D Systems kit. Human EPO levels were below the level of detection (2.5 mU/mL) on day 41 in the plasmid-injected mice.

Figure 7 shows an *in vivo* comparison of circulating hEPO levels in IM versus IV-administered rAAV-hEPO vector, as described in Example 5. Balb/c mice were injected with 3×10^{11} vector genomes IM or IV and serum levels of hEPO were measured by the R and D Systems kit at days 20 and 41.

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Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of virology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al. *Molecular Cloning: A Laboratory Manual* (Current Edition); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed., Current Edition); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds., Current Edition); *Transcription and Translation* (B. Hames & S. Higgins, eds., Current Edition); *CRC Handbook of Parvoviruses*, vol. I & II (P. Tijssen, ed.); *Fundamental Virology*, 2nd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds.)

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The phrase "delivering a gene" or "transferring a gene" refers to methods or systems for reliably inserting foreign DNA into host cells, such as into muscle cells. Such methods can result in transient or long term expression of nonintegrated transferred DNA, extrachromosomal replication and expression of transferred replicons (e.g., episomes), or integration of

transferred genetic material into the genomic DNA of recipients. Gene transfer provides a unique approach for the treatment of acquired and inherited diseases. A number of systems have been developed for gene transfer
5 into mammalian cells. See, e.g., U.S. Patent No. 5,399,346.

The term "therapeutic protein" refers to a protein which is defective or missing from the subject in question, thus resulting in a disease state or disorder
10 in the subject, or to a protein which confers a benefit to the subject in question, such as an antiviral, antibacterial or antitumor function. A therapeutic protein can also be one which modifies any one of a wide variety of biological functions, such as endocrine,
15 immunological and metabolic functions. Representative therapeutic proteins are discussed more fully below.

By "vector" is meant any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, virus, virion, etc., which is capable of replication when
20 associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

By "AAV vector" is meant a vector derived from
25 an adeno-associated virus serotype, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAVX7, etc. AAV vectors can have one or more of the AAV wild-type genes deleted in whole or part, preferably the rep and/or cap genes (described below), but retain functional
30 flanking ITR sequences (also described below). Functional ITR sequences are necessary for the rescue, replication and packaging of the AAV virion. Thus, an AAV vector is defined herein to include at least those sequences required in cis for replication and packaging
35 (e.g., functional ITRs) of the virus. The ITRs need not

be the wild-type nucleotide sequences, and may be altered, e.g., by the insertion, deletion or substitution of nucleotides, so long as the sequences provide for functional rescue, replication and packaging.

5 By "recombinant virus" is meant a virus that has been genetically altered, e.g., by the addition or insertion of a heterologous nucleic acid construct into the particle.

10 By "AAV virion" is meant a complete virus particle, such as a wild-type (wt) AAV virus particle (comprising a linear, single-stranded AAV nucleic acid genome associated with an AAV capsid protein coat). In this regard, single-stranded AAV nucleic acid molecules of either complementary sense, e.g., "sense" or
15 "antisense" strands, can be packaged into any one AAV virion and both strands are equally infectious.

A "recombinant AAV virion," or "rAAV virion" is defined herein as an infectious, replication-defective virus composed of an AAV protein shell, encapsidating a
20 DNA molecule of interest which is flanked on both sides by AAV ITRs. An rAAV virion is produced in a suitable host cell which has had an AAV vector, AAV helper functions and accessory functions introduced therein. In this manner, the host cell is rendered capable of
25 encoding AAV polypeptides that are required for packaging the AAV vector (containing a recombinant nucleotide sequence of interest) into recombinant virion particles for subsequent gene delivery.

The term "transfection" is used to refer to the
30 uptake of foreign DNA by a mammalian cell. A cell has been "transfected" when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are known in the art. See, e.g., Graham et al. (1973) *Virology*, 52:456, Sambrook et al. (1989)
35 *Molecular Cloning, a laboratory manual*, Cold Spring

Harbor Laboratories, New York, Davis et al. (1986) *Basic Methods in Molecular Biology*, Elsevier, and Chu et al. (1981) *Gene* 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties, such as a
5 plasmid vector and other nucleic acid molecules, into suitable host cells. The term refers to both stable and transient uptake of the genetic material.

The term "transduction" denotes the delivery of a DNA molecule to a recipient cell either *in vivo* or *in*
10 *vitro*, via a replication-defective viral vector, such as via a recombinant AAV virion.

By "muscle cell" or "muscle tissue" is meant a cell or group of cells derived from muscle, including but not limited to cells and tissue derived from skeletal
15 muscle; smooth muscle, e.g., from the digestive tract, urinary bladder and blood vessels; and cardiac muscle. The term captures muscle cells both *in vitro* and *in vivo*. Thus, for example, an isolated cardiomyocyte would constitute a "muscle cell" for purposes of the present
20 invention, as would a muscle cell as it exists in muscle tissue present in a subject *in vivo*. The term also encompasses both differentiated and nondifferentiated muscle cells, such as myocytes such as myotubes, myoblasts, both dividing and differentiated,
25 cardiomyocytes and cardiomyoblasts.

The term "heterologous" as it relates to nucleic acid sequences such as gene sequences and control sequences, denotes sequences that are not normally joined together, and/or are not normally associated with a
30 particular cell. Thus, a "heterologous" region of a nucleic acid construct or a vector is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region
35 of a nucleic acid construct could include a coding

16

sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a cell transformed with a construct which is not normally present in the cell would be considered heterologous for purposes of this invention. Allelic variation or naturally occurring mutational events do not give rise to heterologous DNA, as used herein.

By "DNA" is meant a polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in double-stranded or single-stranded form, either relaxed and supercoiled. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes single- and double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having the sequence homologous to the mRNA). The term captures molecules that include the four bases adenine, guanine, thymine, or cytosine, as well as molecules that include base analogues which are known in the art.

A "gene" or "coding sequence" or a sequence which "encodes" a particular protein, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the

gene are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A gene can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA
5 sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the gene sequence.

The term "control elements" refers collectively to promoter regions, polyadenylation signals,
10 transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell.
15 Not all of these control elements need always be present so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell.

The term "promoter region" is used herein in
20 its ordinary sense to refer to a nucleotide region comprising a DNA regulatory sequence, wherein the regulatory sequence is derived from a gene which is capable of binding RNA polymerase and initiating transcription of a downstream (3'-direction) coding
25 sequence.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control elements operably linked to a coding sequence are
30 capable of effecting the expression of the coding sequence. The control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be
35 present between a promoter sequence and the coding

17

sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

For the purpose of describing the relative position of nucleotide sequences in a particular nucleic acid molecule throughout the instant application, such as when a particular nucleotide sequence is described as being situated "upstream," "downstream," "3'," or "5'" relative to another sequence, it is to be understood that it is the position of the sequences in the "sense" or "coding" strand of a DNA molecule that is being referred to as is conventional in the art.

"Homology" refers to the percent of identity between two polynucleotide or two polypeptide moieties. The correspondence between the sequence from one moiety to another can be determined by techniques known in the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs. Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when at least about 80%, preferably at least about 90%, and most preferably at least about 95% of the nucleotides or amino acids match over a defined length of the molecules, as determined using the methods above.

By "mammalian subject" is meant any member of the class *Mammalia* including, without limitation, humans and nonhuman primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and

18

cats; laboratory animals including rodents such as mice, rats and guinea pigs, and the like. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are
5 intended to be covered.

B. General Methods

The present invention provides for the successful transfer of a selected gene to a muscle cell
10 using recombinant AAV virions. The method allows for the direct, *in vivo* injection of recombinant AAV virions into muscle tissue, e.g., by intramuscular injection, as well as for the *in vitro* transduction of muscle cells which can subsequently be introduced into a subject for
15 treatment. The invention also provides for secretion of the produced protein *in vivo*, from transduced muscle cells, such that systemic delivery can be achieved.

Differentiated muscle cells and tissue provide a desirable target for gene therapy since they are
20 readily accessible and nondividing. However, the present invention also finds use with nondifferentiated muscle cells, such as myoblasts, which can be transduced *in vitro*, and subsequently introduced into a subject.

Since muscle has ready access to the
25 circulatory system, a protein produced and secreted by muscle cells and tissue *in vivo* will enter the bloodstream for systemic delivery. Furthermore, since sustained, therapeutic levels of protein secretion from muscle is achieved *in vivo* using the present invention,
30 repetitive exogenous delivery is avoided or reduced in frequency such that therapy can be accomplished using only one or few injections. Thus, the present invention provides significant advantages over prior gene delivery methods.

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The recombinant AAV virions of the present invention, including the DNA of interest, can be produced using standard methodology, known to those of skill in the art. The methods generally involve the steps of (1) introducing an AAV expression vector into a host cell; (2) introducing an AAV helper construct into the host cell, where the helper construct includes AAV coding regions capable of being expressed in the host cell to complement AAV helper functions missing from the AAV vector; (3) introducing one or more helper viruses and/or accessory function vectors into the host cell, wherein the helper virus and/or accessory function vectors provide accessory functions capable of supporting efficient recombinant AAV ("rAAV") virion production in the host cell; and (4) culturing the host cell to produce rAAV virions. The AAV expression vector, AAV helper construct and the helper virus or accessory function vector(s) can be introduced into the host cell, either simultaneously or serially, using standard transfection techniques.

1. AAV Expression Vectors

AAV expression vectors are constructed using known techniques to at least provide as operatively linked components in the direction of transcription, control elements including a transcriptional initiation region, the DNA of interest and a transcriptional termination region. The control elements are selected to be functional in a mammalian muscle cell. The resulting construct which contains the operatively linked components is bounded (5' and 3') with functional AAV ITR sequences.

The nucleotide sequences of AAV ITR regions are known. See, e.g., Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801; Berns, K.I. "Parvoviridae and their

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Replication" in *Fundamental Virology*, 2nd Edition, (B.N. Fields and D.M. Knipe, eds.) for the AAV-2 sequence. AAV ITRs used in the vectors of the invention need not have a wild-type nucleotide sequence, and may be altered, e.g.,

5 by the insertion, deletion or substitution of nucleotides. Additionally, AAV ITRs may be derived from any of several AAV serotypes, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAVX7, etc. Furthermore, 5' and 3' ITRs which flank a selected

10 nucleotide sequence in an AAV expression vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, i.e., to allow for excision and rescue of the sequence of interest from a host cell genome or vector,

15 and to allow integration of the DNA molecule into the recipient cell genome when AAV Rep gene products are present in the cell.

Suitable DNA molecules for use in AAV vectors will be less than about 5 kilobases (kb) in size and will

20 include, for example, a gene that encodes a protein that is defective or missing from a recipient subject or a gene that encodes a protein having a desired biological or therapeutic effect (e.g., an antibacterial, antiviral or antitumor function).

25 Suitable DNA molecules include, but are not limited to, those encoding for proteins used for the treatment of endocrine, metabolic, hematologic, cardiovascular, neurologic, musculoskeletal, urologic, pulmonary and immune disorders, including such disorders

30 as inflammatory diseases, autoimmune, chronic and infectious diseases, such as AIDS, cancer, hypercholesterolemia, insulin disorders such as diabetes, growth disorders, various blood disorders including various anemias, thalassemias and hemophilia; genetic

35 defects such as cystic fibrosis, Gaucher's Disease,

Hurler's Disease, adenosine deaminase (ADA) deficiency, emphysema, or the like.

To exemplify the invention, the gene encoding erythropoietin (EPO) has been used. EPO is a hormone which controls the formation of red blood cells in the bone marrow. The sequence of this gene, as well as methods of obtaining the same, have been described in, e.g., U.S. Patent no. 4,954,437, incorporated herein by reference in its entirety, as well as in Jacobs et al. (1985) *Nature* 313:806-810; Lin et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:7580; International Publication Number WO 85/02610; and European Patent Publication Number 232,034 B1. The recombinant AAV virions described herein which include a gene encoding EPO, or encoding an analog or derivative thereof having the same function, are particularly useful in the treatment of blood disorders characterized by defective red blood cell formation, such as in the treatment of anemia. Increased red blood cell production due to the introduction of the EPO gene can be readily determined by an appropriate indicator, such as by comparing hematocrit measurements pre- and post-treatment. As described above, the EPO gene is flanked by AAV ITRs.

The selected nucleotide sequence, such as EPO or another gene of interest, is operably linked to control elements that direct the transcription or expression thereof in the subject *in vivo*. Such control elements can comprise control sequences normally associated with the selected gene. Alternatively, heterologous control sequences can be employed. Useful heterologous control sequences generally include those derived from sequences encoding mammalian or viral genes. Examples include, but are not limited to, the SV40 early promoter, mouse mammary tumor virus LTR promoter; adenovirus major late promoter (Ad MLP); a herpes simplex

virus (HSV) promoter, a cytomegalovirus (CMV) promoter such as the CMV immediate early promoter region (CMVIE), a rous sarcoma virus (RSV) promoter, synthetic promoters, hybrid promoters, and the like. In addition, sequences
5 derived from nonviral genes, such as the murine metallothionein gene, will also find use herein. Such promoter sequences are commercially available from, e.g., Stratagene (San Diego, CA).

For purposes of the present invention, control
10 elements, such as muscle-specific and inducible promoters, enhancers and the like, will be of particular use. Such control elements include, but are not limited to, those derived from the actin and myosin gene families, such as from the myoD gene family (Weintraub et
15 al. (1991) *Science* 251:761-766); the myocyte-specific enhancer binding factor MEF-2 (Cserjesi and Olson (1991) *Mol. Cell Biol.* 11:4854-4862); control elements derived from the human skeletal actin gene (Muscat et al. (1987) *Mol. Cell Biol.* 7:4089-4099) and the cardiac actin gene;
20 muscle creatine kinase sequence elements (Johnson et al. (1989) *Mol. Cell Biol.* 9:3393-3399) and the murine creatine kinase enhancer (mCK) element; control elements derived from the skeletal fast-twitch troponin C gene, the slow-twitch cardiac troponin C gene and the slow-
25 twitch troponin I gene; hypoxia-inducible nuclear factors (Semenza et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:5680-5684; Semenza et al. *J. Biol. Chem.* 269:23757-23763); steroid-inducible elements and promoters, such as the glucocorticoid response element (GRE) (Mader and
30 White (1993) *Proc. Natl. Acad. Sci. USA* 90:5603-5607); the fusion consensus element for RU486 induction; and elements that provide for tetracycline regulated gene expression (Dhawan et al. (1995) *Somat. Cell. Mol. Genet.* 21:233-240; Shockett et al. (1995) *Proc. Natl. Acad. Sci.*
35 *USA* 92:6522-6526.

These and other regulatory elements can be tested for potential *in vivo* efficacy using the *in vitro* myoblast model, which mimics quiescent *in vivo* muscle physiology, described in the examples below.

- 5 The AAV expression vector which harbors the DNA molecule of interest bounded by AAV ITRs, can be constructed by directly inserting the selected sequence(s) into an AAV genome which has had the major AAV open reading frames ("ORFs") excised therefrom.
- 10 Other portions of the AAV genome can also be deleted, so long as a sufficient portion of the ITRs remain to allow for replication and packaging functions. Such constructs can be designed using techniques well known in the art. See, e.g., U.S. Patent Nos. 5,173,414 and 5,139,941;
- 15 International Publication Nos. WO 92/01070 (published 23 January 1992) and WO 93/03769 (published 4 March 1993); Lebkowski et al. (1988) *Molec. Cell. Biol.* 8:3988-3996; Vincent et al. (1990) *Vaccines 90* (Cold Spring Harbor Laboratory Press); Carter, B.J. (1992) *Current Opinion in*
- 20 *Biotechnology* 3:533-539; Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801; Shelling and Smith (1994) *Gene Therapy* 1:165-169; and Zhou et al. (1994) *J. Exp. Med.* 179:1867-1875.
- 25 Alternatively, AAV ITRs can be excised from the viral genome or from an AAV vector containing the same and fused 5' and 3' of a selected nucleic acid construct that is present in another vector using standard ligation techniques, such as those described in Sambrook et al.,
- 30 *supra*. For example, ligations can be accomplished in 20 mM Tris-Cl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 33 ug/ml BSA, 10 mM-50 mM NaCl, and either 40 uM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C
- 35 (for "blunt end" ligation). Intermolecular "sticky end"

ligations are usually performed at 30-100 $\mu\text{g/ml}$ total DNA concentrations (5-100 nM total end concentration). AAV vectors which contain ITRs have been described in, e.g., U.S. Patent no. 5,139,941. In particular, several AAV
5 vectors are described therein which are available from the American Type Culture Collection ("ATCC") under Accession Numbers 53222, 53223, 53224, 53225 and 53226.

Additionally, chimeric genes can be produced synthetically to include AAV ITR sequences arranged 5' and 3' of one or more selected nucleic acid sequences. Preferred codons for expression of the chimeric gene sequence in mammalian muscle cells can be used. The complete chimeric sequence is assembled from overlapping oligonucleotides prepared by standard methods. See,
10 e.g., Edge, *Nature* (1981) 292:756; Nambair et al. *Science* (1984) 223:1299; Jay et al. *J. Biol. Chem.* (1984) 259:6311.

In order to produce rAAV virions, an AAV expression vector is introduced into a suitable host cell using known techniques, such as by transfection. A
20 number of transfection techniques are generally known in the art. See, e.g., Graham et al. (1973) *Virology*, 52:456, Sambrook et al. (1989) *Molecular Cloning*, a laboratory manual, Cold Spring Harbor Laboratories, New York, Davis et al. (1986) *Basic Methods in Molecular Biology*, Elsevier, and Chu et al. (1981) *Gene* 13:197. Particularly suitable transfection methods include calcium phosphate co-precipitation (Graham et al. (1973) *Viol.* 52:456-467), direct micro-injection into cultured
25 cells (Capecchi, M.R. (1980) *Cell* 22:479-488), electroporation (Shigekawa et al. (1988) *BioTechniques* 6:742-751), liposome mediated gene transfer (Mannino et al. (1988) *BioTechniques* 6:682-690), lipid-mediated transduction (Felgner et al. (1987) *Proc. Natl. Acad.*
30 *Sci. USA* 84:7413-7417), and nucleic acid delivery using

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high-velocity microprojectiles (Klein et al. (1987) *Nature* 327:70-73).

For the purposes of the invention, suitable host cells for producing rAAV virions include microorgan-
isms, yeast cells, insect cells, and mammalian cells,
that can be, or have been, used as recipients of a
heterologous DNA molecule. The term includes the progeny
of the original cell which has been transfected. Thus, a
"host cell" as used herein generally refers to a cell
which has been transfected with an exogenous DNA
sequence. Cells from the stable human cell line, 293
(readily available through, e.g., the American Type
Culture Collection under Accession Number ATCC CRL1573)
are preferred in the practice of the present invention.
Particularly, the human cell line 293 is a human
embryonic kidney cell line that has been transformed with
adenovirus type-5 DNA fragments (Graham et al. (1977) *J.*
Gen. Virol. 36:59), and expresses the adenoviral E1a and
E1b genes (Aiello et al. (1979) *Virology* 94:460). The
293 cell line is readily transfected, and provides a
particularly convenient platform in which to produce rAAV
virions.

2. AAV Helper Functions

Host cells containing the above-described AAV
expression vectors must be rendered capable of providing
AAV helper functions in order to replicate and
encapsidate the nucleotide sequences flanked by the AAV
ITRs to produce rAAV virions. AAV helper functions are
generally AAV-derived coding sequences which can be
expressed to provide AAV gene products that, in turn,
function in trans for productive AAV replication. AAV
helper functions are used herein to complement necessary
AAV functions that are missing from the AAV expression
vectors. Thus, AAV helper functions include one, or both

of the major AAV ORFs, namely the *rep* and *cap* coding regions, or functional homologues thereof.

By "AAV *rep* coding region" is meant the art-recognized region of the AAV genome which encodes the replication proteins Rep 78, Rep 68, Rep 52 and Rep 40. These Rep expression products have been shown to possess many functions, including recognition, binding and nicking of the AAV origin of DNA replication, DNA helicase activity and modulation of transcription from AAV (or other heterologous) promoters. The Rep expression products are collectively required for replicating the AAV genome. For a description of the AAV *rep* coding region, see, e.g., Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; and Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801. Suitable homologues of the AAV *rep* coding region include the human herpesvirus 6 (HHV-6) *rep* gene which is also known to mediate AAV-2 DNA replication (Thomson et al. (1994) *Virology* 204:304-311).

By "AAV *cap* coding region" is meant the art-recognized region of the AAV genome which encodes the capsid proteins VP1, VP2, and VP3, or functional homologues thereof. These Cap expression products supply the packaging functions which are collectively required for packaging the viral genome. For a description of the AAV *cap* coding region, see, e.g., Muzyczka, N. and Kotin, R.M. (*supra*).

AAV helper functions are introduced into the host cell by transfecting the host cell with an AAV helper construct either prior to, or concurrently with, the transfection of the AAV expression vector. AAV helper constructs are thus used to provide at least transient expression of AAV *rep* and/or *cap* genes to complement missing AAV functions that are necessary for productive AAV infection. AAV helper constructs lack AAV

ITRs and can neither replicate nor package themselves. These constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs have been described, such as the
5 commonly used plasmids pAAV/Ad and pIM29+45 which encode both Rep and Cap expression products. See, e.g., Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McCarty et al. (1991) *J. Virol.* 65:2936-2945. A number
10 of other vectors have been described which encode Rep and/or Cap expression products. See, e.g., U.S. Patent No. 5,139,941.

Both AAV expression vectors and AAV helper constructs can be constructed to contain one or more optional selectable markers. Suitable markers include
15 genes which confer antibiotic resistance or sensitivity to, impart color to, or change the antigenic characteristics of those cells which have been transfected with a nucleic acid construct containing the selectable marker when the cells are grown in an
20 appropriate selective medium. Several selectable marker genes that are useful in the practice of the invention include the hygromycin B resistance gene (encoding Aminoglycoside phosphotransferase (APH)) that allows selection in mammalian cells by conferring resistance to
25 G418 (available from Sigma, St. Louis, Mo.). Other suitable markers are known to those of skill in the art.

3. AAV Accessory Functions

The host cell (or packaging cell) must also be
30 rendered capable of providing non AAV derived functions, or "accessory functions," in order to produce rAAV virions. Accessory functions are non AAV derived viral and/or cellular functions upon which AAV is dependent for its replication. Thus, accessory functions include at
35 least those non AAV proteins and RNAs that are required

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in AAV replication, including those involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of Cap expression products and AAV capsid assembly. Viral-based
5 accessory functions can be derived from any of the known helper viruses.

Particularly, accessory functions can be introduced into and then expressed in host cells using methods known to those of skill in the art. Commonly,
10 accessory functions are provided by infection of the host cells with an unrelated helper virus. A number of suitable helper viruses are known, including adenoviruses; herpesviruses such as herpes simplex virus types 1 and 2; and vaccinia viruses. Nonviral accessory
15 functions will also find use herein, such as those provided by cell synchronization using any of various known agents. See, e.g., Buller et al. (1981) *J. Virol.* 40:241-247; McPherson et al. (1985) *Virology* 147:217-222; Schlehofer et al. (1986) *Virology* 152:110-117.

20 Alternatively, accessory functions can be provided using an accessory function vector. Accessory function vectors include nucleotide sequences that provide one or more accessory functions. An accessory function vector is capable of being introduced into a
25 suitable host cell in order to support efficient AAV virion production in the host cell. Accessory function vectors can be in the form of a plasmid, phage, transposon or cosmid. Accessory vectors can also be in the form of one or more linearized DNA or RNA fragments
30 which, when associated with the appropriate control elements and enzymes, can be transcribed or expressed in a host cell to provide accessory functions.

Nucleic acid sequences providing the accessory functions can be obtained from natural sources, such as
35 from the genome of an adenovirus particle, or constructed

using recombinant or synthetic methods known in the art. In this regard, adenovirus-derived accessory functions have been widely studied, and a number of adenovirus genes involved in accessory functions have been

5 identified and partially characterized. See, e.g., Carter, B.J. (1990) "Adeno-Associated Virus Helper Functions," in *CRC Handbook of Parvoviruses*, vol. I (P. Tijssen, ed.), and Muzyczka, N. (1992) *Curr. Topics. Microbiol. and Immun.* 158:97-129. Specifically, early

10 adenoviral gene regions E1a, E2a, E4, VAI RNA and, possibly, E1b are thought to participate in the accessory process. Janik et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:1925-1929. Herpesvirus-derived accessory functions have been described. See, e.g., Young et al. (1979)

15 *Prog. Med. Virol.* 25:113. Vaccinia virus-derived accessory functions have also been described. See, e.g., Carter, B.J. (1990), *supra.*, Schlehofer et al. (1986) *Virology* 152:110-117.

As a consequence of the infection of the host

20 cell with a helper virus, or transfection of the host cell with an accessory function vector, accessory functions are expressed which transactivate the AAV helper construct to produce AAV Rep and/or Cap proteins. The Rep expression products excise the recombinant DNA

25 (including the DNA of interest) from the AAV expression vector. The Rep proteins also serve to duplicate the AAV genome. The expressed Cap proteins assemble into capsids, and the recombinant AAV genome is packaged into the capsids. Thus, productive AAV replication ensues,

30 and the DNA is packaged into rAAV virions.

Following recombinant AAV replication, rAAV virions can be purified from the host cell using a variety of conventional purification methods, such as CsCl gradients. Further, if infection is employed to

35 express the accessory functions, residual helper virus

can be inactivated, using known methods. For example, adenovirus can be inactivated by heating to temperatures of approximately 60°C for, e.g., 20 minutes or more. This treatment effectively inactivates only the helper
5 virus since AAV is extremely heat stable while the helper adenovirus is heat labile.

The resulting rAAV virions are then ready for use for DNA delivery, such as in gene therapy applications, for the production of transgenic animals,
10 in vaccination, and particularly for the delivery of genes to a variety of muscle cell types.

4. In vitro and In vivo Delivery of rAAV Virions

Generally, rAAV virions are introduced into a
15 muscle cell using either *in vivo* or *in vitro* transduction techniques. If transduced *in vitro*, the desired recipient muscle cell will be removed from the subject, transduced with rAAV virions and reintroduced into the subject. Alternatively, syngeneic or xenogeneic muscle
20 cells can be used where those cells will not generate an inappropriate immune response in the subject.

Suitable methods for the delivery and introduction of transduced cells into a subject have been described. For example, cells can be transduced *in vitro*
25 by combining recombinant AAV virions with muscle cells e.g., in appropriate media, and screening for those cells harboring the DNA of interest using conventional techniques such as Southern blots and/or PCR, or by using selectable markers. Transduced cells can then be
30 formulated into pharmaceutical compositions, described more fully below, and the composition introduced into the subject by various techniques, such as by intramuscular, intravenous, subcutaneous and intraperitoneal injection, or by injection into smooth and cardiac muscle, using
35 e.g., a catheter.

For *in vivo* delivery, the rAAV virions will be formulated into pharmaceutical compositions and will generally be administered parenterally, e.g., by intramuscular injection directly into skeletal or cardiac muscle.

Pharmaceutical compositions will comprise sufficient genetic material to produce a therapeutically effective amount of the protein of interest, i.e., an amount sufficient to reduce or ameliorate symptoms of the disease state in question or an amount sufficient to confer the desired benefit. The pharmaceutical compositions will also contain a pharmaceutically acceptable excipient. Such excipients include any pharmaceutical agent that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991).

Appropriate doses will depend on the mammal being treated (e.g., human or nonhuman primate or other mammal), age and general condition of the subject to be treated, the severity of the condition being treated, the particular therapeutic protein in question, its mode of administration, among other factors. An appropriate

effective amount can be readily determined by one of skill in the art.

Thus, a "therapeutically effective amount" will fall in a relatively broad range that can be determined through clinical trials. For example, for *in vivo* injection, i.e., injection directly to skeletal or cardiac muscle, a therapeutically effective dose will be on the order of from about 10^6 to 10^{15} of the rAAV virions, more preferably 10^8 to 10^{12} rAAV virions. For *in vitro* transduction, an effective amount of rAAV virions to be delivered to muscle cells will be on the order of 10^8 to 10^{13} of the rAAV virions. The amount of transduced cells in the pharmaceutical compositions will be from about 10^4 to 10^{10} muscle cells, more preferably 10^5 to 10^8 muscle cells. When the transduced cells are introduced to vascular smooth muscle, a lower dose may be appropriate. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves.

Dosage treatment may be a single dose schedule or a multiple dose schedule. Moreover, the subject may be administered as many doses as appropriate. One of skill in the art can readily determine an appropriate number of doses.

25

C. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

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Materials and MethodsVector constructsA. Construction of p1909adh_{lacZ}.

Plasmid p1909adh_{lacZ} was used as the helper
5 construct in the following examples and was constructed
from plasmid pWadh_{lacZ}. Plasmid pWadh_{lacZ} was
constructed by partially digesting plasmid pUC119
(GeneBank Reference Name: U07649, GeneBank Accession
Number: U07649) with *Afl*III and *Bsp*HI, blunt-end
10 modifying with the klenow enzyme, and then ligating to
form a circular 1732 bp plasmid containing the bacterial
origin and the *amp* gene only (the polylinker and *F1*
origin was removed). The blunted and ligated *Afl*III and
*Bsp*HI junction forms a unique *Nsp*I site. The 1732 bp
15 plasmid was cut with *Nsp*I, blunt-end modified with T4
polymerase, and a 20 bp *Hin*DIII-*Hinc*II fragment (blunt-
end modified with the klenow enzyme) obtained from the
pUC119 polylinker was ligated into the blunted *Nsp*I site
of the plasmid. The *Hin*DIII site from the blunted
20 polylinker was regenerated, and then positioned adjacent
to the bacterial origin of replication. The resulting
plasmid was then cut at the unique *Pst*I/*Sse*8387I site,
and an *Sse*8387I-*Pvu*II-*Sse*8387I oligonucleotide
(5'-GGCAGCTGCCTGCA-3')^(SEQ ID NO. 1) was ligated in. The remaining
25 unique *Bsp*HI site was cut, blunt-end modified with klenow
enzyme, and an oligonucleotide containing an *Asc*I linker
(5'-GAAGGCGCGCCTTC-3')^(SEQ ID NO. 2) was ligated therein, eliminating
the *Bsp*HI site. The resulting plasmid was called pWee.

In order to create the pWadh_{lacZ} construct, a
30 CMV_{lacZ} expression cassette (comprising a nucleotide
sequence flanked 5' and 3' by AAV ITRs, containing the
following elements: a CMV promoter, the hGH 1st intron,
an adh_{lacZ} fragment and an SV40 early polyadenylation
site) was inserted into the unique *Pvu*II site of pWee

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using multiple steps such that the CMV promoter was arranged proximal to the bacterial *amp* gene of pWee.

More particularly, a CMVlacZ expression cassette was derived from the plasmid psub201CMV, which was constructed as follows. An oligonucleotide encoding the restriction enzyme sites:

NotI-MluI-SnaBI-AgeI-BstBI-BssHII-NcoI-HpaI-BspEI-PmlI-RsrII-NotI

and having the following nucleotide sequence:

10 5'-GCGGCCGCACGCGTACGTACCGGTTCTGAAGCGCGCACGGCCGACCATGGTTAAC
TCCGGACACGTGCGGACCGCGGCCGC-3' (SEQ ID No.: 3) was
synthesized and cloned into the blunt-end modified
KasI-EarI site (partial) of pUC119 to provide a 2757 bp
vector fragment. A 653 bp SpeI-SacII fragment containing
15 a nucleotide sequence encoding a CMV immediate early
promoter was cloned into the SnaBI site of the 2757 bp
vector fragment. Further, a 269 bp PCR-produced
BstBI-BstBI fragment containing a nucleotide sequence
encoding the hGH 1st intron which was derived using the
20 following primers:
5'-AAAATTCGAACCTGGGGAGAAACCAGAG-3' (SEQ ID NO.: 4) and
3'-aaaattcgaacaggtgaagcgcccctTTG-5' (SEQ ID NO.: 5), was
cloned into the BstBI site of the 2757 bp vector
fragment, and a 135 bp HpaI-BamHI (blunt-end modified)
25 fragment containing the SV40 early polyadenylation site
from the pCMV- β plasmid (CLONETECH) was cloned into the
HpaI site of the subject vector fragment. The resulting
construct was then cut with NotI to provide a first CMV
expression cassette.

30 Plasmid pW1909adh_{lacZ} was constructed as follows. A 4723 bp SpeI-EcoRV fragment containing the
AAV rep and cap encoding region was obtained from the
plasmid pGN1909 (ATCC Accession Number 69871). The
pGN1909 plasmid is a high efficiency AAV helper plasmid
35 having AAV rep and cap genes with an AAV p5 promoter

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region that is arranged in the construct to be downstream from its normal position (in the wild type AAV genome) relative to the rep coding region. The 4723 bp fragment was blunt-end modified, and AscI linkers

5 (5'-GAAGGCGCGCCTTC-3')^(SEQ ID NO. 2) were ligated to the blunted ends. The resultant fragment was then ligated into the unique AscI site of pWadhIacZ and oriented such that the AAV coding sequences were arranged proximal to the bacterial origin of replication in the construct.

10 Plasmid pW1909adhIacZ includes the bacterial beta-galactosidase (β -gal) gene under the transcriptional control of the cytomegalovirus immediate early promoter (CMVIE).

15 B. Construction of pW1909EPO.

Plasmid pW1909adhIacZ was modified to express human erythropoietin (EPO) by replacing the adhIacZ gene with a 718 bp PpuMI-NcoI fragment of human EPO cDNA and by cloning a 2181 bp ClaI-EcoRI lacZ spacer fragment
20 (noncoding) into the PmlI site of the vector.

Viruses and Cell Lines

Adenovirus type 2 (Ad2), available from the American Type Culture Collection, ATCC, Catalogue Number
25 VR846, was used as helper virus to encapsidate vectors.

The human 293 cell line (Graham et al. (1977) *J. Gen. Virol.* 36:59-72, available from the ATCC under Accession no. CRL1573), which has adenovirus E1a and E1b genes stably integrated in its genome, was cultured in
30 complete Dulbecco's modified Eagle's media (DMEM; Bio-Whittaker, Walkersville, Maryland) containing 4.5 g/l glucose, 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, Utah), 2mM glutamine, and 50 units/ml penicillin and 50 μ g/ml streptomycin.

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The C2C12 murine myoblast cell line, available from the ATCC, Catalogue Number CRL1772, was cultured in complete DME.

5 The above cell lines were incubated at 37°C in 5% CO₂, and were routinely tested and found free of mycoplasma contamination.

Cardiomyocytes were prepared by a modification of established methods. In particular, primary rat myocardial cell isolation was done by modifying
10 established protocols by Nag and Chen (1981) *Tissue Cell* 13:515-523 and Dlugaz et al. (1984) *J. Cell Biol.* 99:2268-2278. Briefly hearts from newborn rat pups (one or two litters) were dissected and washed in media. Digestion media consisted of modified Jolicks MEM
15 containing 10 mM HEPES, 10 mM pyruvate, 5 mM L-glutamine, 1 mM Nicotinamide, 0.4 mM L-ascorbate, 1 mM adenosine, 1 mM d-ribose, 1 mM MgCl₂, 1 mM taurine, 2 mM DL-carnitine, and 2 mM KHCO₃. The hearts were minced in digestion
20 media with 0.5 mg/ml collagenase (Worthington) and 100 mM CaCl₂. The tissue was treated with successive digestions of 15 minutes at 37°C. The cells from the first digestion were discarded and the next six digestions
25 reactions were pooled. Cells were preplated for 1 hour to remove fibroblasts, then plated in PC-1 (Ventrex)/DME-Hams F12 media.

Production of Recombinant AAV Virions

Recombinant AAV virions were produced in human 293 cells as follows. Subconfluent 293 cells were
30 cotransfected by standard calcium phosphate precipitation with either vector/helper plasmid constructs pW1909adhLacZ or pW1909EPO. Cells were infected with Ad2 at a multiplicity of infection (MOI) of 2, and incubated
35 at 37°C in 5% CO₂ for 70 hours prior to harvest. Cells were lysed in Tris buffer (100mM Tris, 150mM NaCl, pH

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8.0), freeze-thawed three times, then crude-cell lysate was layered onto a cesium chloride cushion for isopycnic gradient centrifugation. Recombinant AAV vectors were extracted from the resulting gradient by isolating the
5 bands with average density of approximately 1.38 g/ml, resuspending in Hepes buffered saline (HBS) containing 50mM Hepes, 150mM NaCl, pH 7.4, and heat-inactivating the preparation at 56°C for 1 hour.

10 Assay of rAAV by Dot-blot Hybridization

Recombinant AAV virions were DNase I digested, proteinase K treated, phenol-chloroform extracted, and DNA precipitated with sodium acetate-glycogen (final concentrations = 0.3M sodium acetate and 160 µg/ml,
15 respectively). DNA samples were denatured (200 µl of 2X alkaline solution (0.8 M NaOH, 20 mM EDTA) added to DNA sample) for 10 minutes, then added to appropriate wells in a dot-blot apparatus, and blotted to wet Zeta Probe membrane (BioRad), by applying suction until wells were
20 empty. Then, 400 µl of 1X alkaline solution was added; after 5 minutes, wells were emptied by suction. The membrane was rinsed in 2X SSC (Sambrook et al., supra) for 1 min, drained, air dried on filter paper, then baked in vacuum at 80°C for 30 min. The membrane was then
25 prehybridized for 30 min at 65°C with 10 ml hybridization buffer (7% SDS, 0.25 M Sodium Phosphate, pH 7.2, 1 mM EDTA). Buffer was replaced with 10 ml fresh solution, freshly boiled probe added, and hybridized overnight at 65°C. The membrane was washed twice with 25 ml of wash-1
30 buffer (5% SDS, 40mM sodium phosphate, pH 7.2, 1mM EDTA) for 20 min at 65°C and twice with wash-2 buffer (1% SDS, 40mM sodium phosphate, pH 7.2, 1mM EDTA). The membrane was wrapped in plastic film, exposed to radiographic film, and appropriate dots excised from the membrane to
35 determine radioactivity by scintillation counting, and

quantitated by comparison with standards. Titers of rAAV vector were routinely in the range of approximately 10^{13} vector genomes/ml.

5 Assay for Contaminating Helper Adenovirus

Contaminating infectious adenovirus was assayed as follows. Samples from the purified rAAV virion stocks were added to 50% confluent 293 cells (cultured in 12 well dishes at 1×10^5 cells/well), and the cultures were
10 passed for 30 days (e.g., the cultures were split 1 to 5, every 3 days) or until the culture exhibited 100% cytopathic effect (CPE) due to adenovirus infection. Cultures were examined daily for CPE, and the day upon which each experimental culture showed 100% CPE was
15 noted. Reference 293 cell cultures infected with a range of known amounts of adenovirus type-2 (from 0 to 1×10^7 plaque forming units (pfu)/culture) were also prepared and treated in the same manner. A standard curve was then prepared from the data obtained from the reference
20 cultures, where the adenovirus pfu number was plotted against the day of 100% CPE. The titer of infectious adenovirus type-2 in each experimental culture was then readily obtained as determined from the standard curve. The limit of detection of the assay was 100 pfu/ml. The
25 presence of wild-type AAV contamination, analyzed by dot-blot hybridization, was approximately 7 logs lower than vector concentration.

In vitro Transduction

30 In vitro transduction was performed by adding purified recombinant AAV virions to 293 or C2C12 cells in complete media, incubating for a designated period of time, usually a minimum of 24 hours, prior to the determination of transduction efficiency, by β -gal, or
35 human EPO (hEPO) assays. Histochemical detection of the

presence of β -gal was done by a previously reported technique (Sanes et al. (1986) *EMBO J* 5:3133-3142).

The hEPO assay was performed by an enzyme-linked immunosorbance assay (ELISA) using the human erythropoietin Quantikine IVD kit from R and D Systems (Minneapolis, MN) according to manufacturer's recommendations.

C2C12 myoblasts were transduced either while actively dividing, or as a differentiated cell culture. Differentiation was induced by placing subconfluent myoblasts in DMEM containing 2% horse serum and standard concentrations of glutamine and penicillin-streptomycin for an interval of four days prior to transduction. Verification of differentiation was by microscopic analysis to determine the presence of multinucleated myotubes in culture.

Methods of in vivo Transduction of Murine Skeletal Muscle

In vivo transduction was performed by intramuscular (IM) injection of recombinant AAV virions into the skeletal muscle of adult Balb/c mice (Jackson Laboratories, Bar Harbor, ME, Simonsen Laboratories, Gilroy, CA, or Harlan Laboratories) under either Metofane (Pitman-Moore, Mundelein, IL) or ketamine-xylazine anesthesia. For the rAAV-LacZ dose-response and time-course, the head of the tibialis anterior muscle was isolated under anesthesia, and injected 2 mm deep with a micro-capillary tube to administer 10-20 μ l of rAAV in saline. For the rAAV-EPO dose-response and time-course, different doses of the vector diluted in HBS were injected percutaneously into 3 sites in each hindlimb, 100 μ l total vector per hindlimb, for a total of 200 μ l of vector IM/animal. Animals were warmed prior to returning them to their cages.

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Analysis of in vivo Transduction

For analysis of gene delivery with rAAV-LacZ, muscle was harvested at various time points post-injection, cryofixed, and stained for β -gal or for Galacto-LightTM (Tropix, Bedford, MA) according to manufacturer's recommendations. B-gal stained tibialis anterior cross-sections were photomicrographed, and scored as positive or negative to quantify the percentage of cross-sectional muscle fiber transduced. For Galacto-LightTM assay, the muscle was homogenized and mixed with substrate containing buffer to produce a luminescent product, which was quantified by luminometer.

For analysis of gene delivery with rAAV-hEPO in Balb/c mice, vector was administered either IM as described above, or intravenously (IV) in PBS in a total volume of 50 μ l via the lateral tail vein. At various time points after administration, blood was obtained from the orbital venous plexus under anesthesia. Red cell counts were done with hemocytometer, hematocrit was determined by centrifugation of blood in micro-capillary tubes, and hemoglobin concentration was analyzed by cyanmethemoglobin assay (DMA, Arlington, TX) according to manufacturer's specifications and compared with a standard (Stanbio Laboratory, San Antonio, TX) analyzed at 570 nm on a spectrophotometer. Reticulocytes were analyzed by either new methylene blue stain, or by FACS analysis of thiazole orange stained peripheral blood samples (Retic-count, Becton-Dickinson, Mountain View, CA); the results of data obtained by either of these methods were similar. Peripheral leukocyte count and differential were performed by modified Wright-Giemsa stain (Sigma Diagnostics, St. Louis, MO) according to the manufacturer's recommendations.

Intramyocardial Injections

For the cardiac muscle studies, animals were anesthetized with Metofane followed by a subxyphoid incision to expose the diaphragmatic surface of the heart. Apical cardiac injections were performed with a glass micropipette. Recombinant virion was diluted in normal saline and injected in volume of 20-50 μ l.

Histochemical Analysis of Cardiac Muscle

For 5-Bromo-4-chloro-3-indolyl B-D-galactoside histochemical determination, frozen sections (6 μ m) were fixed in 0.5% glutaraldehyde and stained for β -gal activity as described (Sanes et al. (1986) "Use of Recombinant Retrovirus to Study Post-Implantation Cell Lineage in Mouse Embryos," *EMBO J* 5:3133-3142).

Histopathology

Paraffin sections (5 μ m) were stained with hematoxylin/eosin. Sections were examined for infiltrating mononuclear cells.

Example 1

Dose-Response of rAAV-LacZ Vector in Balb/c Mice

To determine the effective dose range for the rAAV-LacZ vector *in vivo*, vector was injected into the tibialis anterior muscle of 6-8 week old healthy Balb/c mice, and transduction assessed by β -gal activity measured by Galacto-Light™ relative light units (RLU). Two weeks post-injection, the range of RLU is from approximately 0.2 x 10⁷ RLU/muscle with 8 x 10⁸ vector genomes to approximately 1.1 x 10⁹ RLU/muscle with 3.6 x 10¹¹ injected vector genomes (Figure 1). The levels of expression of β -gal measured by RLU correspond to the percentage of β -gal positive muscle fibers on cross-sectional analysis. For example, 0.2 x 10⁷ RLU

corresponds to approximately 1 % β -gal positive muscle fibers; 1.1×10^9 RLU corresponds to approximately 60 % β -gal positive muscle fibers. Expression of a gene delivered by a rAAV vector was approximately 10,000-fold greater than the same gene delivered by plasmid DNA. A comparison was made at two weeks post-injection of rAAV-LacZ vector, 8×10^8 single-stranded genomes, with plasmid (containing the same gene sequence as the vector), 2.4×10^{13} double-stranded plasmid genomes in 100 μ g DNA, a high dose of plasmid DNA IM. Results revealed comparable expression between vector and plasmid, however vector input DNA was approximately 4 logs less. These results demonstrate the dose-dependent expression of β -gal by rAAV-LacZ and the greatly increased expression of rAAV vector versus plasmid.

Example 2

Time Course of rAAV-LacZ Vector Expression in vivo

Animals were followed after injection to determine the persistence of vector expression. For these experiments, animals were injected in the tibialis anterior muscle with 8×10^9 vector genome equivalents of rAAV-LacZ in 20 μ l volume. Muscle was harvested at selected time points from 2 to 12 weeks and analyzed for total β -gal expression by Galacto-Light™ luminescence. As can be seen, the level of β -gal production increased over the time interval from 2 to 12 weeks post-injection (Figure 2). These results demonstrate that rAAV-LacZ expression in muscle persisted for an interval of at least 12 weeks in vivo.

Example 3Comparison of Secretion of Erythropoietin
from Myotubes or Myoblasts

Myotubes (differentiated C2C12 cells) or
5 myoblasts (dividing C2C12 cells) were transduced in
culture with the rAAV-hEPO vector to determine the
feasibility of *in vivo* polypeptide secretion.
Erythropoietin (EPO) was chosen because it has been shown
to be secreted by muscle (Descamps et al. (1995) *Gene*
10 *Therapy* 2:411-417; Hamamori et al. (1994) *Hum. Gene*
Therapy 5:1349-1356; Hamamori et al. (1995) *J. Clin.*
Invest. 95:1808-1813) and it has well defined biological
effects. A comparison of the secretion of EPO from
myotubes or myoblasts revealed that secretion of the
15 hormone was observed in both populations. Additionally,
the levels of 24 hour EPO secretion increased in the
myotubes over the first seven days post-transduction
(Figure 3). These data demonstrate that gene transfer of
rAAV-hEPO into either myotubes or myoblasts results in
20 protein secretion.

Example 4Systemic Delivery of Human Erythropoietin *in vivo*
by Intramuscular Administration of rAAV-hEPO

25 Recombinant AAV virions encoding hEPO were
administered to adult healthy Balb/c mice *in vivo* to
determine if hEPO was produced and was biologically
active. An initial experiment revealed that high levels
of hEPO and elevated hematocrits were maintained for >
30 100 days in mice injected IM with 6.5×10^{11} vector
genomes (Figure 4). A range of doses of rAAV-hEPO was
then injected into the hindlimbs of mice, and the
resulting serum hEPO levels were analyzed. A well-
defined dose-response at both 20 and 41 days post-
35 injection is revealed in Figure 5, with the levels of

hEPO dependent upon input vector dose. Expression of a gene delivered by rAAV-hEPO vector was approximately 6000-fold greater than the same gene delivered by plasmid DNA (Figure 6). A comparison of the expression of IM rAAV-hEPO (3×10^{11} single-stranded vector genomes) with IM pAAV-hEPO (1.4×10^{13} double-stranded genomes in $100\mu\text{g}$ DNA) was performed. At 20 days post-injection, vector-injected animals had serum levels of 445.1 ± 98.1 mU/ml while the plasmid-injected animals had levels of 7.7 ± 10 mU/ml. At 41 days post-injection, the vector levels were 724.6 ± 112 mU/ml, while the plasmid levels were below the levels of detection. The animals receiving rAAV-hEPO exhibited approximately 60-fold more circulating hEPO with 100-fold less input genomes at 20 days post-injection, or approximately 6000-fold greater secretion per genome. At 41 days post-injection, this difference was even greater, since the plasmic expression was below the level of detection.

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Example 5A Comparison of hEPO Secretion from rAAV-hEPOAdministered by IM or IV Routes

A comparison of the circulating levels of hEPO resulting from IM and IV routes of administration was analyzed to determine which method of gene delivery results in higher levels of systemic hEPO. At 20 days post-injection, the IM route resulted in levels of hEPO of 445.1 ± 98.1 mU/ml while the IV route produced 6.5 ± 3.0 mU/ml (Figure 7). At 41 days post-injection, the EPO level for the IM route was 724.6 ± 112 compared with 13.0 ± 2.0 mU/ml, or approximately 60-fold more efficacious. These data demonstrate that the IM route of injection resulted in higher systemic levels of hEPO.

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Example 6

Expression of rAAV-LacZ in Terminally
Differentiated Adult Rat Cardiomyocytes

The ability of recombinant AAV vectors to
5 transduce terminally differentiated adult cardiomyocytes
was established *in vivo*. Cardiomyocytes were harvested
by coronary perfusion with collagenase of adult rat
hearts (Fischer 344, Harlan Sprague Dawley, Indianapolis,
IN). Cardiomyocytes were grown on laminin-coated glass
10 coverslips and exposed to the rAAV-LacZ vector for 4
hours. After 72 hours, the cells were stained for β -gal
activity. AAV expression was detected by blue staining
of the binucleated cells. These studies demonstrate the
ability of recombinant AAV virions to transduce
15 terminally differentiated cells. The transduction
efficiency *in vitro* was 30% of adult cells at a
multiplicity of infection of 10^4 vector genomes per cell.

Example 7

20 Stability of LacZ Expression *In Vivo*

Adult Fischer rats were used to analyze
expression of transgenes *in vivo*. Incremental doses of
rAAV-LacZ were injected into the left ventricular apex of
the rat heart accessed using either the subxyphoid or
25 lateral thoracotomy approaches. At varying times post-
injection, the hearts were harvested and examined for β -
gal production. Greater than 50% transduction of
cardiomyocytes was observed in the region of injection at
each time point examined. There was no inflammatory cell
30 infiltrate noted during the course of analysis. B-gal
staining was observed to persist in cardiac muscle for at
least two months following gene transfer.

Accordingly, novel methods for transferring
genes to muscle cells have been described. Although
35 preferred embodiments of the subject invention have been

described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

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Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strain was made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, under the provisions of the Budapest Treaty. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, lose its plasmid, it will be replaced with a viable culture(s) of the same taxonomic description.

This deposit is provided merely as a convenience to those of skill in the art, and is not an admission that a deposit is required. A license may be required to make, use, or sell the deposited materials, and no such license is hereby granted.

	<u>Strain</u>	<u>Deposit Date</u>	<u>ATCC No.</u>
30	pGN1909	July 20, 1995	69871

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